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Lipid-Dependent Modulation of Ca^{2+} Availability in Isolated Mossy Fiber Nerve Endings*

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An enhancement of glutamate release from hippocampal neurons has been implicated in long-term potentiation, which is thought to be a cellular correlate of learning and memory. This phenomenom appears to be involved the activation of protein kinase C and lipid second messengers have been implicated in this process. The purpose of this study was to examine how lipid-derived second messengers, which are known to potentiate glutamate release, influence the accumulation of intraterminal free Ca^{2+}, since exocytosis requires Ca^{2+} and a potentiation of Ca^{2+} accumulation may provide a molecular mechanism for enhancing glutamate release. The activation of protein kinase C with phorbol esters potentiates the depolarization-evoked release of glutamate from mossy fiber and other hippocampal nerve terminals. Here we show that the activation of protein kinase C also enhances evoked presynaptic Ca^{2+} accumulation and this effect is attenuated by the protein kinase C inhibitor staurosporine. In addition, the protein kinase C-dependent increase in evoked Ca^{2+} accumulation was reduced by inhibitors of phospholipase A_2 and voltage-sensitive Ca^{2+} channels, as well as by a lipoxygenase product of arachidonic acid metabolism. That some of the effects of protein kinase C activation were mediated through phospholipase A_2 was also indicated by the ability of staurosporine to reduce the Ca^{2+} accumulation induced by arachidonic acid or the phospholipase A_2 activator melittin. Similarly, the synergistic facilitation of evoked Ca^{2+} accumulation induced by a combination of arachidonic acid and diacylglycerol analogs was attenuated by staurosporine. We suggest, therefore, that the protein kinase C-dependent potentiation of evoked glutamate release is reflected by increases in presynaptic Ca^{2+} and that the lipid second messengers play a central role in this enhancement of chemical transmission processes.

KEY WORDS: Arachidonic acid; diacylglycerol; protein kinase C; phospholipase A_2, calcium; glutamate.

INTRODUCTION

The depolarization-dependent increase in the concentration of intraterminal free Ca^{2+} ($[Ca^{2+}]_i$) stimulates the release of neurotransmitters and it is likely that any potentiation of the evoked increase in $[Ca^{2+}]_i$ can facilitate neurotransmission by enhancing transmitter exocytosis. This enhanced efflux of transmitter, in particular glutamate, has been implicated in the potentiation of synapses thought to be involved in learning and memory (1). The activation of protein kinase C (PKC) has also been linked to synaptic potentiation (2) and the associated increase in evoked glutamate release (3,4,5). Therefore, it is possible that this potentiation of glutamate exocytosis depends on PKC activation and results in an increase in $[Ca^{2+}]_i$.

There are several factors known to activate PKC. First, phorbol esters translocate PKC to cellular mem-

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branes, in a relatively permanent manner, where it phosphorylates substrates (6). This translocation/activation of PKC appears to play a neuromodulatory role, since 4-β-phorbol 12,13-dibutyrate (PDBu) enhances depolarization-evoked glutamate release and presynaptic Ca^{2+} accumulation (4). In addition, lipid second messengers activate PKC. Diacylglycerol (DAG), formed as a result of the breakdown of phosphatidylinositol 4,5-bisphosphate, activates PKC by lowering its affinity for Ca^{2+} (7). Similarly, arachidonic acid (AA), liberated from the *sn*-2 position of phospholipids by phospholipase A_2 (PLA_2), stimulates PKC (8). Also, a role for lipids in PKC-dependent synaptic potentiation is indicated by the observations that exogenous AA, or the PLA_2-dependent accumulation of AA, enhances glutamate efflux from nerve terminals (9), as well as an increase in $[Ca^{2+}]_i$ (10). More recently, it was shown that a combination of low concentrations of AA and DAG activates PKC (11) and potentiates the evoked efflux of glutamate from nerve terminals (5) in a synergistic manner. The purpose of the work presented here was to examine the relationships between the lipid messengers, PKC activation, presynaptic Ca^{2+} accumulation and the facilitation of glutamate exocytosis.

Hippocampal mossy fiber synaptosomes (HMFS) were used as the model for the following studies, since this preparation contains the presynaptic elements of the mossy fiber-CA3 synapse and this synapse expresses a potentiation that is dependent on the facilitation of glutamate exocytosis (12). HMFS respond to depolarizing stimuli with increases in $[Ca^{2+}]_i$ and glutamate efflux (13,14). In addition, the synaptosomes contain PKC isoforms, some of which are activated by phorbol esters (4) and they accumulate unesterified AA upon depolarization (14). Here it is shown that the activation of PKC leads to a potentiation of Ca^{2+} accumulation, whether induced by membrane depolarization or an ionophore. In addition, inhibition of PKC attenuated the increase in evoked Ca^{2+} accumulation induced by phorbol esters, unesterified AA or the PLA_2 activator melittin. A physiological role for AA in presynaptic processes was indicated by the observation that low concentrations of the fatty acid were able to enhance depolarization-evoked Ca^{2+} accumulation when DAG analogs were present. Again, this effect was attenuated by PKC inhibition. It was also observed that certain receptor agonists mimicked the effects of exogenous DAG analogs on Ca^{2+} availability and glutamate exocytosis. Based on these observations, we suggest that the short-term effects of PKC activation on glutamate exocytosis may be linked to an enhancement in presynaptic Ca^{2+} concentrations and that the lipid-derived second messengers are involved in this facilitation.

EXPERIMENTAL PROCEDURE

Preparation of Hippocampal Mossy Fiber Synaptosomes. Six to eight adult Sprague-Dawley rats (150 – 200 g) were used to obtain mossy fiber synaptosomes. Details of this procedure have been described (13). The final pellet was resuspended in oxygenated Krebs-bicarbonate buffer (122 mM NaCl, 3.1 mM KCl, 1.2 mM $MgSO_4$, 0.4 mM KH_2PO_4, 5 mM $NaHCO_3$, 20 mM sodium N-tris[hydroxymethyl]-methyl-2-aminoethane-sulfonic acid (Na-TES), 11 mM D-glucose; pH 7.4). Aliquots were taken to determine protein concentration using the Warburg-Christian assay (15).

Measurement of Intracellular Ca^{2+}. Synaptosomes were split into 5 mg aliquots in Ca^{2+}-free Krebs-bicarbonate buffer containing 0.5% fatty acid free bovine serum albumin and incubated in a shaking water bath at 30°C for 5 minutes to remove unesterified fatty acids and facilitate the movement of fura-2AM, a fluorescent Ca^{2+} dye, into the synaptosomes (16). Synaptosomes were pelleted in a microfuge, the supernatant was removed and the synaptosomes were washed once with Krebs-bicarbonate buffer. Fura-2AM (5 μM) was loaded into the synaptosomes for 20 min at 30°C. During this incubation period cytosolic esterases hydrolyze the acetoxymethyl esters of fura-2AM trapping the dye inside the synaptosomes. Synaptosomes were centrifuged as previously mentioned and washed twice in Krebs-bicarbonate buffer to remove any external dye. Synaptosomes were resuspended in oxygenated (95% O_2, 5% CO_2) Krebs-Ringer buffer (128 mM NaCl, 5 KCl, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM Na_2HPO_4, 10 mM D-glucose and 20 mM MOPS; pH 7.4) and incubated at 30°C for 15 min to allow for Ca^{2+} equilibration within the nerve terminals.

Following incubation, aliquots of synaptosomes (0.25 mg protein) were added to a quartz cuvette containing Krebs-Ringer buffer and placed in a Perkin Elmer LS-5B spectrofluorimeter. Synaptosomes were maintained in suspension throughout the experiment with a magnetic stir bar. Experimental treatments were added directly to the cuvette 2 min prior to depolarization. All treatments were checked for autofluorescence and treatments diluted with DMSO were added such that the total concentration of DMSO in the cuvette did not exceed 0.1%. This concentration of DMSO did not affect Ca^{2+} measurements.

The concentration of intracellular Ca^{2+} was determined using a ratio method (17), which has been routinely employed in this laboratory (18,10). R_{max} was determined by lysing the cells with 0.1% sodium dodecylsulfate (SDS) and R_{min} was determined by binding the available Ca^{2+} with 8 mM EGTA. Results are shown as nM changes in the concentrations of intraterminal free Ca^{2+} from resting levels.

Glutamate Release. Isolated nerve terminals were superfused in 6 parallel chambers maintained at 30°C at a rate of 0.5 mL/min. Four-minute fractions were collected and assayed for glutamate using a microfluorometric assay (19) as described (9,13). Spontaneous glutamate efflux was determined by superfusion with control buffer. Evoked release was calculated as the difference between spontaneous efflux and the total amount of glutamate released in the presence of stimuli. Evoked release is expressed as picomoles of glutamate released per minute per mg of protein.

The lipid second messengers and most other special chemicals used in this study were obtained from the Sigma Chemical Company. Fura-2AM was purchased from Molecular Probes, while norepinephrine bitartrate was obtained from Research Biochemicals Inc.

Statistical Analyses. Data are expressed as the means obtained from multiple experiments ± SEM. Statistical significance among treatment groups was determined using ANOVA and the Newman-Keuls pairwise comparison test. A value is considered significant at $p < 0.05$.

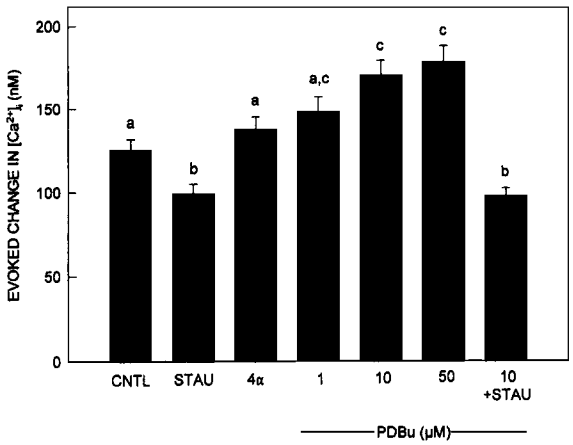

Fig. 1. Effects of 4β-phorbol 12,13-dibutyrate (PDBu) on K^+-evoked Ca^{2+} accumulation in the absence or presence of staurosporine (STAU; 1 μM). HMFS were prepared and $[Ca^{2+}]_i$ was measured as described. Synaptosomes were exposed to each treatment for 2 min prior to depolarization with 25 mM KCl. CNTL = depolarization (25 mM K^+) without treatment. 4α = 4α-phorbol (10 μM). Results are expressed as the nM evoked changes in $[Ca^{2+}]_i$ ± SEM. Different letters represent significantly different values; $p < 0.05$.

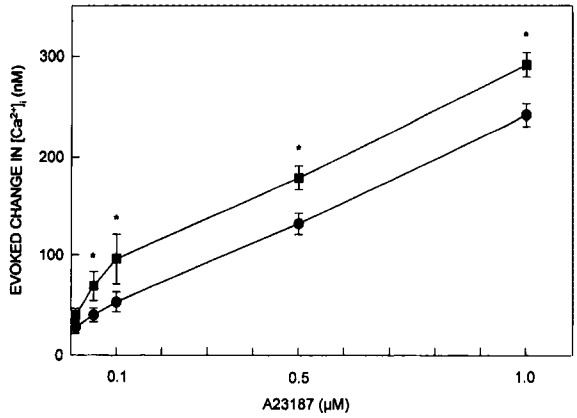

Fig. 2. 8-Bromo-A23187 (A23187)-stimulated increases in $[Ca^{2+}]_i$ in the absence or presence of PDBu. Nerve terminals were prepared and $[Ca^{2+}]_i$ was quantified as described. Synaptosomes were exposed to A23187 at the concentrations shown in the absence (closed circles) or presence (closed squares) of PDBu (10 μM) preincubation for 2 min. Results are shown as nM stimulated changes in $[Ca^{2+}]_i$ ± SEM. * = significantly different from concentration-matched A23187 controls; $p < 0.05$.

Table I. Effects of PLA_2 Inhibitors, 12-HETE and Ca^{2+} Channel Blockers on K^+-Evoked Ca^{2+} Accumulation in the Presence of 10 μM PDBu

Treatment	Evoked change in $[Ca^{2+}]_i$ (nM)	% Inhibition
Control	129 ± 8.5*	—
PDBu (10 μM)	170 ± 9.3	—
+ 4-BPB (50 μM)	91 ± 9.0*	44
+ NDGA (100 μM)	80 ± 8.0*	53
+ 12-HETE (10 μM)	76 ± 7.6*	55
+ Verapamil (300 μM)	39 ± 9.3*	77
+ Cinnarizine (20 μM)	32 ± 8.0*	81

HMFS were prepared and $[Ca^{2+}]_i$ was determined as described. Treatments were applied to the nerve terminals 2 min before depolarization with 25 mM KCl. Results are expressed as the evoked nM change in $[Ca^{2+}]_i$ from resting levels ± SEM in the first column and as the % of inhibition from PDBu-enhanced evoked Ca^{2+} accumulation in the second column. PDBu = 4β-phorbol 12,13-dibutyrate, 4-BPB = 4-bromophenacyl bromide, NDGA = nordihydroguaiaretic acid, 12-HETE = 12-S-hydroxyeicosatetraenoic acid, * = significantly different from the PDBu-enhanced evoked change in $[Ca^{2+}]_i$; $p < 0.05$.

RESULTS

Role of PKC in Depolarization-Evoked Ca^{2+} Accumulation. The resting level of intraterminal free Ca^{2+} in mossy fiber nerve terminals was 338 ± 5.2 nM. Depolarization with 25 mM KCl stimulated a 126 nM increase in $[Ca^{2+}]_i$ which was inhibited 21% by the protein kinase inhibitor, staurosporine (Fig. 1). PDBu potentiated the evoked accumulation of Ca^{2+} in a dose-dependent manner and this enhancement was blocked by staurosporine (1 μM). The inactive 4α-phorbol had no effect on evoked Ca^{2+} accumulation.

The PDBu-enhanced Ca^{2+} accumulation was attenuated by the PLA_2 inhibitors, 4-bromophenacyl bromide (4-BPB; 50 μM) and nordihydroguaiaretic acid (NDGA; 100 μM), by 44 or 53%, respectively (Table I). The lipoxygenase product of AA metabolism, 12-S-hydroxyeicosatetraenoic acid (12-HETE; 10 μM), inhibited the K^+-evoked PDBu-enhanced Ca^{2+} accumulation by 55%. In addition, the voltage-sensitive Ca^{2+} channel (VSCC) blockers verapamil (300 μM) or cinnarizine (20 μM) inhibited Ca^{2+} accumulation by 77 or 81%, respectively.

Effects of PDBu on Ca^{2+} Ionophore-Induced Ca^{2+} Accumulation. The Ca^{2+} ionophore, 8-Bromo-A23187 (A23187), stimulated a dose-dependent increase in the accumulation of intraterminal free Ca^{2+}, since 0.01, 0.05, 0.1, 0.5 or 1 μM A23187 increased $[Ca^{2+}]_i$ by 28, 40, 53, 132 or 242 nM, respectively. PDBu (10 μM) potentiated these ionophore-induced increases in $[Ca^{2+}]_i$ by 43, 72, 81, 37 or 21%, respectively (Fig. 2).

Effects of PDBu and Staurosporine on Ca^{2+} Accumulation Induced by Melittin or AA. The PLA_2 activator melittin stimulated a dose-dependent increase in $[Ca^{2+}]_i$, since 0.01, 0.05 or 0.1 μM melittin increased $[Ca^{2+}]_i$ by 22, 48 or 88 nM (Fig. 3). This effect was not altered by 10 μM PDBu. However, 0.2 μM staurosporine inhibited the Ca^{2+} accumulation induced by 0.05 or 0.1 μM melittin by 52 or 22%, respectively. Likewise, AA stimulated a dose-dependent enhancement of Ca^{2+} accumulation which was unaffected by 10 μM PDBu

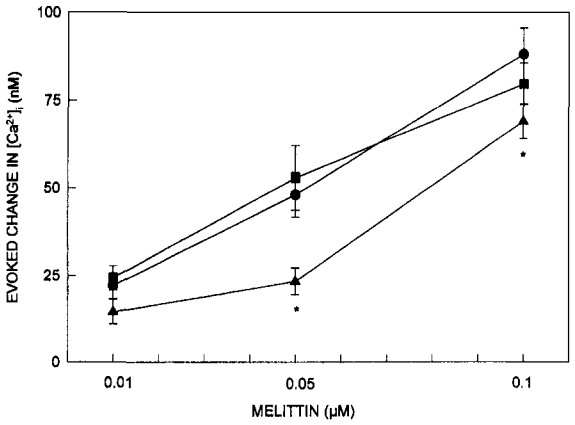

Fig. 3. Effects of PDBu on melittin-induced Ca^{2+} accumulation in the absence or presence of staurosporine. Synaptosomes were isolated as described and Ca^{2+} accumulation is expressed as the nM change ± SEM. Nerve terminals were exposed to melittin at the concentrations given (closed circles). Melittin-treated synaptosomes were exposed to 10 μM PDBu (closed squares) or 0.2 μM staurosporine (closed triangles) for 2 min prior to stimulation with melittin. * = significantly different from the melittin alone value; $p < 0.05$.

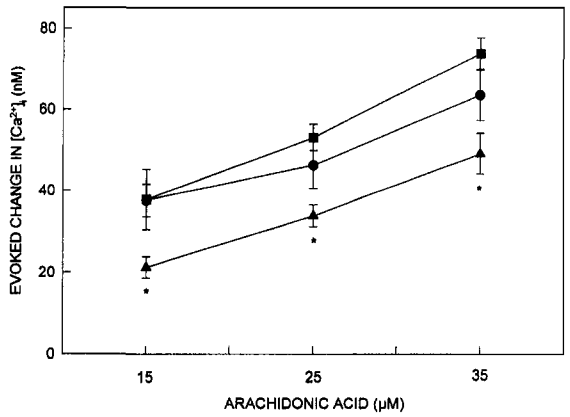

Fig. 4. Effects of PDBu on arachidonic acid (AA)-induced Ca^{2+} accumulation in the absence or presence of staurosporine. HMFS were prepared and $[Ca^{2+}]_i$ was determined as described. HMFS were stimulated with AA at the concentrations shown (closed circles). AA treated synaptosomes were exposed to 10 μM PDBu (closed squares) or 0.2 μM staurosporine (closed triangles) for 2 min prior to exposure to AA. Results are expressed as the stimulated change in $[Ca^{2+}]_i$ ± SEM. * = significantly different from the AA alone value; $p < 0.05$.

(Fig. 4). However, 0.2 μM staurosporine attenuated this accumulation by 44, 37 or 34% when the nerve terminals were stimulated with 15, 25 or 35 μM AA, respectively.

Synergistic Facilitation of Depolarization-Induced Ca^{2+} Accumulation by AA and OAG. The effects of AA and the DAG analog oleoyl-acetyl-glycerol (OAG) on depolarization-evoked intraterminal free Ca^{2+} accumulation were investigated and the results are shown in Fig. 5. Neither OAG (1 μM) nor AA (1 μM) had any effect

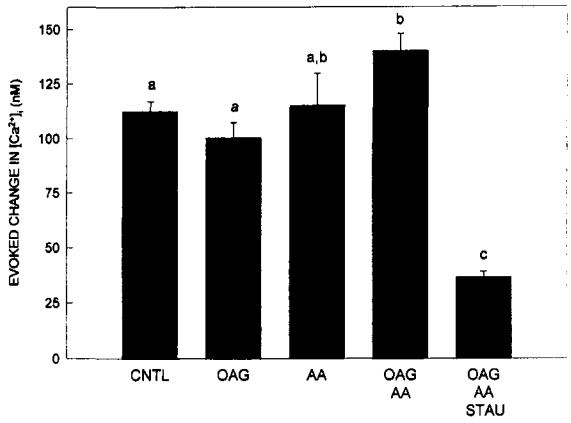

Fig. 5. Effects of staurosporine on arachidonic acid/oleyol-acetyl-glycerol-dependent facilitation of K^+-evoked Ca^{2+} accumulation. Nerve terminals were prepared and $[Ca^{2+}]_i$ was determined as described. HMS were exposed to combinations of STAU (1 μM), oleoyl-acetyl-glycerol (OAG; 1 μM), or AA (1 μM), for 2 min prior to depolarization with 25 mM KCl. CNTL = depolarization without treatment. Results are expressed as nM changes in $[Ca^{2+}]_i$ ± SEM. Different letters represent significantly different values; $p < 0.05$.

on K^+-evoked Ca^{2+} accumulation. However, the presence of a combination of OAG and AA enhanced evoked Ca^{2+} accumulation by 25% and this effect was attenuated 74% by 1 μM staurosporine. Neither OAG nor AA affected the resting $[Ca^{2+}]_i$ at the concentrations used. Similar results were obtained using a less permeable analog of DAG, 1,2-dioctanoyl-sn-glycerol (DiC_8). Here, the presence of 0.01, 0.1 or 0.5 μM DiC_8 had no effect on K^+-evoked Ca^{2+} accumulation, but the same concentrations enhanced evoked Ca^{2+} accumulation by 17, 21, or 30% in the presence of 1 μM AA, respectively.

Effects of Receptor Agonists and AA on Depolarization-Evoked Ca^{2+} Accumulation and Glutamate Release. Several receptor agonists were used in an effort to stimulate the production of endogenous DAG, which may potentiate the evoked accumulation of intraterminal free Ca^{2+} in the presence of AA. Norepinephrine (NE) in the presence of 1 μM AA enhanced evoked Ca^{2+} accumulation by 22% (Fig. 6A). However, NE alone increased $[Ca^{2+}]_i$ by 7% over the depolarization evoked control. NE alone also caused an increase in K^+-evoked glutamate release to 23% above the control. Although this value was increased to 34% of the control in the presence of AA, the difference was not statistically significant from evoked glutamate release potentiated by NE alone (Fig. 6B).

Acetylcholine (ACh) in the presence of 1 μM AA stimulated an 18% increase in evoked Ca^{2+} accumulation over that seen with ACh alone (Fig. 7A). Although, this increase was not significantly different than the K^+-

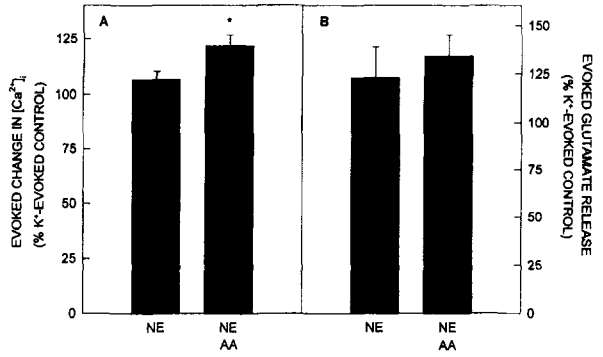

Fig. 6. Effects of NE plus AA on K^+-evoked Ca^{2+} accumulation and glutamate release. Nerve terminals were prepared as described. **Panel A**: $[Ca^{2+}]_i$ was determined as mentioned previously. The HMFS were exposed to NE (10 μM) with or without AA (1 μM) for 2 min prior to depolarization with 25 mM KCl. Results are shown as the depolarization-evoked change in $[Ca^{2+}]_i$ for each treatment divided by the K^+-evoked stimulus control and expressed as % ± SEM. * = significantly different from stimulus control (25 mM K^+); $p < 0.05$. **Panel B**: The efflux of glutamate was determined as described. Nerve terminals were exposed to NE (5 μM) with or without AA (50 μM) for 4 min prior to a 2 min pulse of 25 mM KCl. Results are shown as % stimulus control ± SEM.

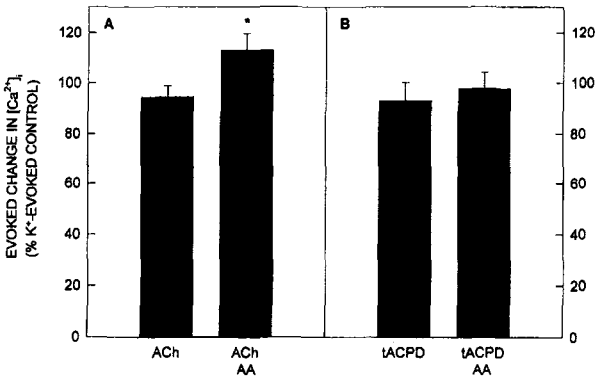

Fig. 7. Effects of ACh or tACPD, in the presence or absence of AA, on K^+-evoked Ca^{2+} accumulation. HMFS were prepared as described and $[Ca^{2+}]_i$ was determined as mentioned previously. The nerve terminals were exposed to ACh (10 μM; **Panel A**) or tACPD (1 μM; **Panel B**) with or without AA (1 μM) for 2 min prior to depolarization with 25 mM KCl. Results are shown as % stimulus control ± SEM. * = significantly different from depolarized, ACh treated value; $p < 0.05$.

evoked Ca^{2+} accumulation in stimulus controls, it was different from the evoked Ca^{2+} accumulation observed in the presence of ACh alone.

The metabotropic glutamate receptor agonist t-ACPD had no influence on evoked Ca^{2+} accumulation at concentrations ranging from 0.1 to 1 μM. The lack of effect at 1 μM is shown in the presence or absence of 1 μM AA (Fig. 7B).

DISCUSSION

The potentiation of the mossy fiber/CA3 synapse appears to involve an increase in evoked glutamate efflux (12) and synaptic potentiation has been associated with increased Ca^{2+} accumulation (20). Ultimately, these effects may involve PKC, since activation of neuronal PKC facilitates Ca^{2+}-dependent glutamate release from cerebrocortical and mossy fiber synaptosomes (3,4). In turn, DAG and AA have been shown to synergistically activate PKC (21,22), and potentiate synapses in the CA1 region of the hippocampus (23). These two second messengers have also been found to synergistically facilitate glutamate release from mossy fiber nerve terminals (5). The purpose of our investigation was to determine if factors which activate PKC, and in effect enhance glutamate release, also lead to an increase in $[Ca^{2+}]_i$.

We observed that the activation of PKC potentiated depolarization-evoked Ca^{2+} accumulation and that PLA_2 inhibitors, a lipoxygenase product and Ca^{2+} channel blockers attenuated this effect. Also, the ionophore-dependent increase in $[Ca^{2+}]_i$ was potentiated by PDBu, but the Ca^{2+} accumulation induced by AA or melittin was not enhanced by the phorbol ester. However, DAG analogs and AA did induce a synergistic facilitation of evoked Ca^{2+} accumulation that apparently required PKC activation. In addition, ACh and NE were able to mimic the effects of the DAG analogs, while tACPD did not. Taken together, these results implicate the lipid second messengers in the PKC-dependent modulations of presynaptic Ca^{2+} concentrations.

The influence of PDBu on depolarization-evoked Ca^{2+} accumulation was consistent with the findings of other groups (3,4,24). The PKC inhibitor staurosporine significantly reduces glutamate release from depolarized nerve terminals exposed to PDBu for 5 min (24). This attenuation of glutamate release may be due to effects on Ca^{2+} concentrations, since we observed that staurosporine caused a 43% reduction in evoked Ca^{2+} accumulation in the presence of PDBu. It should be noted, however, that staurosporine also attenuated depolarization-evoked Ca^{2+} accumulation in the absence of PDBu and this may be due to the inhibition of previously activated mossy fiber PKC (4).

It has been suggested that PKC-dependent phosphorylation stimulates PLA_2 and leads to AA accumulation, which would further enhance cellular processes. For example, the activation of glutamate receptors in brain cortical cultures leads to the Ca^{2+}- and PKC-dependent activation of PLA_2 (25). Similarly, PKC-dependent phosphorylation has been implicated in the

activation of PLA_2 in macrophages (26), smooth muscle (27) and PC 12 cells (28). Here we show that the PLA_2 inhibitors, 4-BPB and NDGA, reduced the effects of PDBu on Ca^{2+} accumulation, suggesting a role for PKC in the activation of mossy fiber PLA_2, as well as some involvement of the lipase in the regulation of $[Ca^{2+}]_i$.

These inhibitors also attenuate K^+-evoked Ca^{2+} accumulation in the absence of PDBu and, based on comparisons with past results (14,18), 4-BPB appears to be equally effective at reducing K^+-evoked Ca^{2+} accumulation in the presence or absence of PDBu. However, NDGA is less effective at attenuating the PDBu effects, perhaps because it also inhibits lipoxygenase activity, which potentiates both evoked glutamate release and Ca^{2+} accumulation (14,18). Consistent with this suggestion, we observed that the lipoxygenase product 12-HETE reduced the PDBu-dependent facilitation of evoked Ca^{2+} accumulation by 55%, which is three-fold greater than the reduction observed in the absence of PDBu (18). Taken together, these data implicate AA in the PKC-dependent effects on $[Ca^{2+}]_i$, and again, one of its lipoxygenase products appeared to attenuate neurotransmission processes. These results are consistent with the recent suggestion that 12-HETE is involved in the development of long-term depression of hippocampal synapses (29).

We also report that PDBu enhanced the Ca^{2+} accumulation induced by the Ca^{2+} ionophore A23187. This effect on $[Ca^{2+}]_i$ was consistent with the observation that PKC activation also potentiates ionophore-induced glutamate release (30). It was suggested that this facilitation may depend on the ability of Ca^{2+} ionophores to depolarize neuronal membranes (31) and open voltage-gated Ca^{2+} channels. This proposal was supported by the partial attenuation of ionophore-induced glutamate release from mossy fiber terminals by VSCC blockers (30).

Although PDBu enhanced the Ca^{2+} accumulation induced by depolarization and A23187, it did not increase that due to melittin or AA. Perhaps AA and PDBu modulated Ca^{2+} accumulation through similar mechanisms. In fact, AA elicits an increase in $[Ca^{2+}]_i$ in mossy fiber nerve terminals (10) and part of this effect may depend on the activation of PKC, since *cis*-unsaturated fatty acids activate the kinase in the absence of Ca^{2+} and phospholipid (8). As with phorbol esters (32), free fatty acids also are likely to regulate PKC at the membrane surface, where they increase lipid fluidity (33), which serves to stabilize PKC (34). Thus, some of the AA effects are likely to be mediated through the activation of PKC. Indeed, staurosporine attenuated the effects of both AA and melittin on $[Ca^{2+}]_i$.

Although, the high concentrations of AA required to directly stimulate PKC may not be physiologically relevant (35) and are likely to be involved in ischemia-induced brain damage (36,37), roles for AA in normal neuronal functions have been proposed. Recently, it was shown that low concentrations of AA activate PKC, but only in the presence of DAG (11,21). Consistent with the formation of an intrinsically active form of PKC (38), the combination of AA and DAG induces synaptic potentiation (23,39) and enhances evoked glutamate release from HMFS (5). It appeared, from the results presented here, that this potentiation of neurotransmitter release was due to an increase in $[Ca^{2+}]_i$, since DAG and AA synergistically enhanced depolarization-evoked Ca^{2+} accumulation. That these effects are attenuated by staurosporine and mimicked by PDBu provides strong evidence for PKC involvement in the modulation of both Ca^{2+} availability and glutamate release.

Several receptor agonists were used in attempts to stimulate the production of endogenous DAG. We employed tACPD, NE and ACh, since the hippocampus is rich in glutamatergic synapses and receives adrenergic and cholinergic input from various brain regions (40). NE enhanced depolarization-evoked Ca^{2+} accumulation by itself and the presence of AA caused a further increase in $[Ca^{2+}]_i$. NE also stimulated glutamate release from resting synaptosomes and its effects on K^+-evoked glutamate efflux showed a facilitory trend. These results were consistent with the ability of NE to enhance the magnitude, duration and probability of synaptic potentiation at the mossy fiber synapse (41), which is likely to occur through the enhancement of Ca^{2+} influx via voltage-gated Ca^{2+} channels (42).

The other receptor agonists, ACh and tACPD, stimulate the production of DAG (43,44) via the activation of PLC. ACh is thought to have a neuromodulatory effect on hippocampal pyramidal cells by stimulating a slow depolarization via the inhibition of K^+ efflux (45). Presynaptically, ACh has been shown to potentiate glutamate release in the presence of AA (30). Hence, evoked Ca^{2+} accumulation displayed a similar pattern, since ACh enhanced depolarization-evoked Ca^{2+} accumulation in the presence of AA. Thus, the activation of ACh receptors may lead to the PLC-dependent production of DAG, which potentiates Ca^{2+} accumulation when AA is added.

Finally, several groups reported the metabotropic glutamate receptor agonists and AA show synergistic effects on neurotransmission. For example, tACPD enhances glutamate release from cerebrocortical synaptosomes and HMFS, but only in the presence of AA (27,46). Similarly, tACPD and AA enhance synaptic

transmission in hippocampal slices from the CA1 region in the absence of tetanic stimulation (47). These effects may involve the modulation of $[Ca^{2+}]_i$, since depolarization-evoked Ca^{2+} accumulation is potentiated by 50 μM tACPD, but only in the presence of 2 μM AA (46). However, we observed no change in the K^+-evoked Ca^{2+} accumulation upon treatment with tACPD and AA. The discrepancy between these findings and those mentioned previously could be due to a highly localized increase in Ca^{2+} influx into the mossy fiber nerve endings, which might not be detected using the current techniques. Alternatively, the primary influence of the lipid second messengers may be mediated through other PKC-dependent processes, such interactions with cytoskeletal components, which potentiate evoked glutamate release by increasing the availability of synaptic vesicles (48).

In summary, activation of PKC stimulated an enhancement of evoked Ca^{2+} accumulation and evidence is provided that PLA_2 may be involved in this PKC-dependent potentiation of evoked Ca^{2+} accumulation. One can speculate that pre- and/or postsynaptic PLA_2 becomes activated in the course of synaptic potentiation and this leads to an increase in presynaptic AA. This AA stimulates the activation of PKC, but only when receptor-derived DAG is present. This activation of PKC affects both $[Ca^{2+}]_i$ and glutamate release. The potentiation of Ca^{2+} accumulation and glutamate exocytosis may be functionally related, but further work is needed to confirm this. Also in question are the receptor subtypes which are coupled to DAG formation, the mechanisms for the control and localization of PLA_2 and the involvement of Ca^{2+} in all these processes.

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